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BULLETIN

OF

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A METHOD FOR THE DIFFERENTIAL STAINING OF GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA IN TISSUE SECTIONS

J. HOWARD BROWN AND LENA BRENN

From the Department of Pathology and Bacteriology, Johns Hopkins University Medical School, Baltimore, Md.

The methods which have been described for the differential staining of Gram-positive and Gram-negative bacteria in tissue sections have often been found unsatisfactory in that the Gram-negative bacteria are apt not to be sharply stained or differentiated from certain tissue elements. In our hands the following method has given excellent results:

Paraffin sections are prepared as usual for staining.

1. Stain in freshly filtered alum-hematoxylin (Harris) for two to five minutes.
2. Wash in acid alcohol (3 per cent HCl in 95 per cent alcohol) until light pink.
3. Wash in ammonia water (1 cc. of aqua ammoniae in 100 cc. water) until blue.
4. Wash in water.
5. In a small vial mix 5 drops of 5 per cent aqueous solution of sodium bicarbonate (containing also 0.5 per cent phenol as a preservative) with about 0.75 cc. of 1 per cent (by weight) aqueous solution of gentian violet. Immediately pour the mixture onto the slide and stain for two minutes.
6. Wash quickly with water.
7. Cover with Lugol's iodine solution for 1 minute.
8. Wash with water. Blot.
9. Decolorize in 1 part of ether plus 3 parts of acetone, dropping it onto the slide until no more color comes off.
10. Blot.
11. Stain for 5 minutes with rosanilin hydrochloride (0.005 gm. per 100 cc. water) or basic fuchsin (0.1 cc. saturated alcoholic solution per 100 cc. water).
12. Wash in water. Blot but do not allow the section to dry.
13. Pass through acetone.
14. Decolorize and differentiate by dropping over the section a solution of 0.1 gram picric acid in 100 cc. of acetone until the section becomes a yellowish-pink. This is the most critical stage of the process and should be carried out by holding the slide over a white plate or dish. Most of the fuchsin should be decolorized from the tissue but the Gram-negative bacteria should remain red.

15. Pass successively through acetone, equal parts of acetone and xylol, and xylol.

16. After clearing in xylol mount in balsam.

(Beginning with step 5 it is best to work with only one slide at a time.)

Cell nuclei should be stained dark reddish-brown; cytoplasm yellowish; Gram-positive bacteria deep violet or almost black; Gram-negative bacteria bright red.

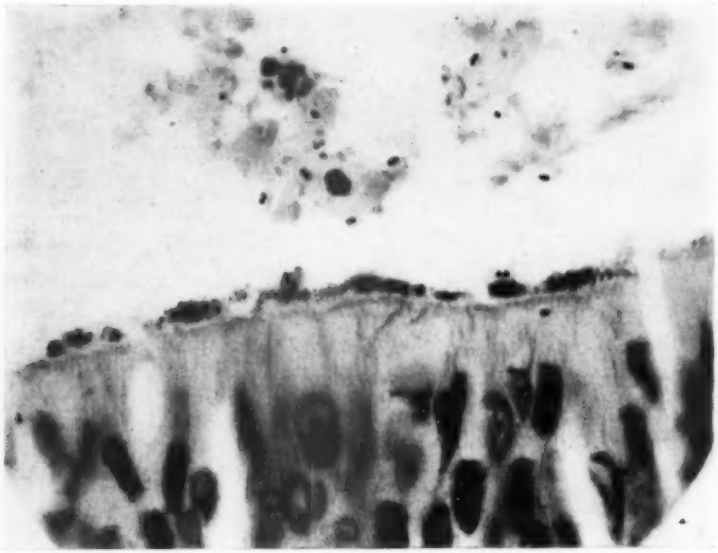
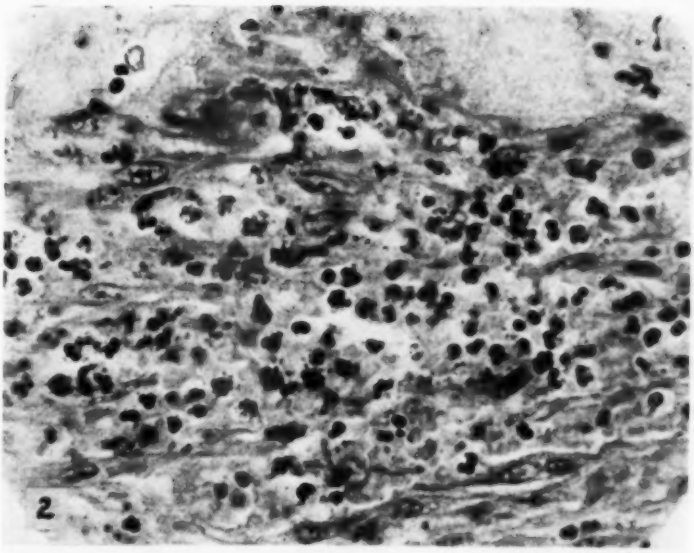


FIG. 1. TRACHEA OF A CHILD

Diagnosis, pertussis. Bordet-Gengou bacilli among the cilia of the epithelial cells and scattered in the exudate. Pneumococci in the exudate. Zenker fixation. Magnification × 1000. Photographed through Eastman B and F color filters.

Leucocytes generally stand out plainly with a dusky yellowish cytoplasm. Basophilic granules stain red. Red blood cells may be yellow or red depending upon the degree of decolorization in picric acid. Cartilege stains pink. Striated muscle and fibrin generally stain yellow but may retain more or less of the red stain. The tissue stain has many of the characteristics of Van Gieson's stain.

By this method we have been able to stain successfully sections of tissue showing influenza bacilli, pneumococci and streptococci; per-



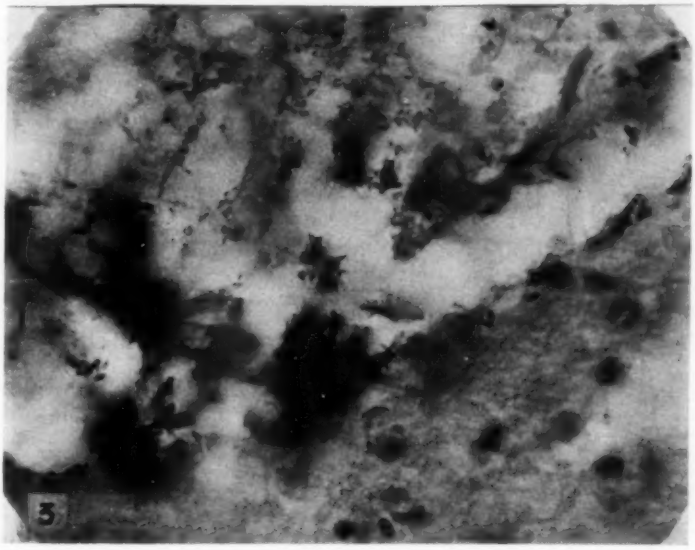
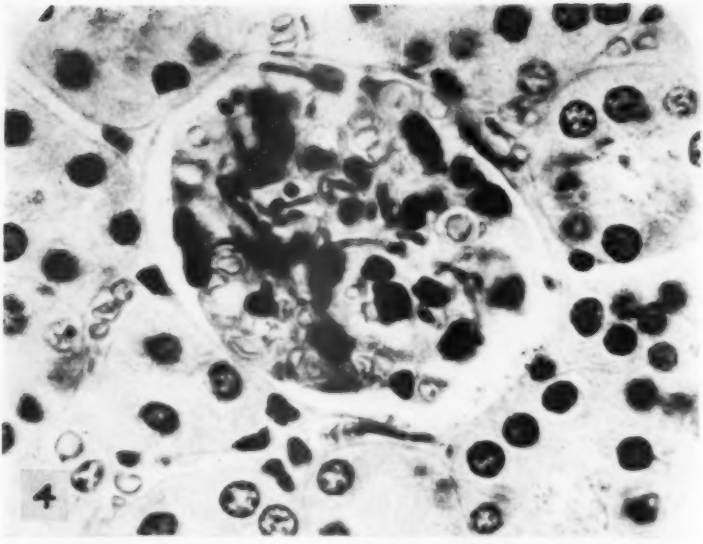


FIG. 2. HUMAN HEART

Diagnosis, gonococcus endocarditis. Magnification × 490. Photographed through Eastman B color filter.

FIG. 3. SPLEEN OF ADULT

Diagnosis, endometritis, peritonitis and septicemia following abortion. Gram-positive bacilli, streptococci and small Gram-negative bacilli (not identified). Zenker fixation. Magnification × 1000. Photographed through Eastman B and F color filters.



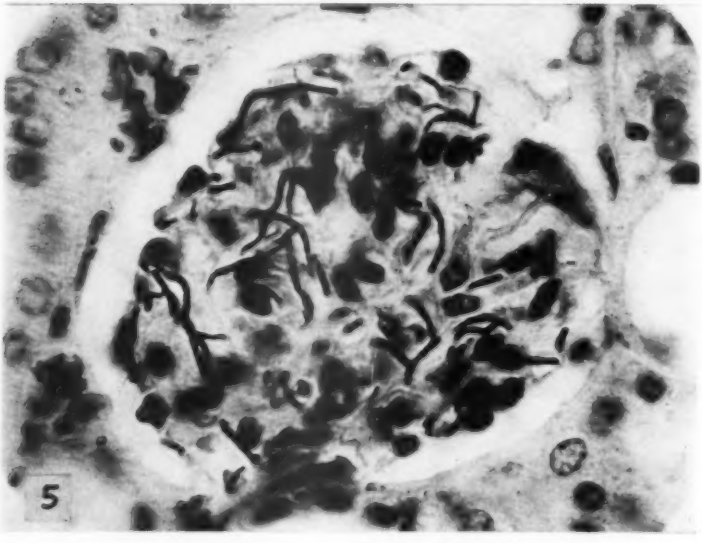


FIG. 4. GLOMERULUS OF KIDNEY OF MOUSE INOCULATED WITH BACILLUS ANTHRACIS

Bacilli stained Gram-negatively. Zenker fixation. Magnification × 1000. Photographed through Eastman B and F color filters.

FIG. 5. GLOMERULUS OF KIDNEY OF MOUSE INOCULATED WITH BACILLUS ANTHRACIS

Bacilli stained Gram-positively. Regaud fixation. Magnification × 1000. Photographed through Eastman B and F color filters.

tussis bacilli crowded among the cilia of the epithelial cells of the trachea and bronchi; gonococci, staphylococci and streptococci in heart valve vegetations; meningococci in the meninges; Corynebacterium pyogenes, Actinomyces necrophorus, Bacillus anthracis, Clostridium welchii and Clostridium septique in animal tissues.

The Gram stain employed in the method as described above is Burke's (1) modification but Stirling's anilin gentian violet or Hucker's ammonium oxalate gentian violet may be used with almost as good results. Using Burke's Gram stain Churchman (2) found that Bacillus anthracis stained Gram-negatively in tissues fixed in Zenker's fluid and embedded in paraffin. We obtained similar results with this organism and with a strain of encapsulated staphylococcus. This result was obtained whether the Zenker's fluid was made up with acetic acid or formalin. However, in bits of the same tissues fixed in 10 per cent formalin or in Regaud's fluid (four parts of 3 per cent aqueous solution of potassium bichromate and one part of formalin) these organisms stained Gram-positively (*cf.*, Figures 4 and 5). The anaërobic bacteria, Clostridium welchii and Clostridium septique, in tissues fixed in Zenker's fluid also showed a great preponderance of Gram-negative organisms. Similar tissues fixed in Regaud's fluid showed more Gram-positive organisms, and tissues fixed in formalin showed a great preponderance of Gram-positive organisms. Even in the formalin fixed tissues, however, it was noted that the bacteria which showed definite evidence of disintegration stained Gram-negatively. With the exception of the encapsulated staphylococcus mentioned above all of the staphylococci, streptococci, pneumococci and diphtheroid bacilli have stained Gram-positively in tissues regardless of the method of fixation employed. Aspergillus was not well stained in tissues.

The method described is useful as a routine stain for bacteria in tissues and is particularly valuable in the case of mixed infections where both Gram-positive and Gram-negative bacteria may be found and differentiated.

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2. Churchman, John W.: Effect of hardening and fixation on Gram reaction, *Proc. Soc. Exp. Biol. and Med.*, 1928, xxv, 336.

THE PRODUCTION OF ACUTE NEPHRITIS BY MEANS OF A PNEUMOCOCCAL AUTOLYSATE[1]

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From the Department of Pathology and Bacteriology, Johns Hopkins University, Baltimore

The problem of the production of nephritis in animals by means of bacterial infection or bacterial products has occupied for some years the attention of many investigators. Their results for the most part have been negative or unconvincing. It is the purpose of this paper to report experiments, still in progress, in which all the changes comparable to those seen in acute and subacute nephritis in man have been produced in rabbits by the use of an autolysate obtained from pneumococci, and also by the intradermal infection with pneumococci. An investigation of our human autopsy records has shown that mild acute and subacute nephritis often unrecognized clinically, together with a few cases of acute florid nephritis, can be demonstrated in about 40 to 50 per cent of those dying from pneumococcal infections, the lesions found in the kidneys often paralleling those produced in our rabbits in a remarkable manner.[2]

In the past the attention of investigators has been devoted almost exclusively to the streptococcus, whether of the α or the β type, as the cause of acute nephritis due to infection. By the use of living streptococci, changes resembling more or less those seen in human nephritis have been produced by Ophüls (1) in rabbits, by Bell, Clawson, and Hartzell (2) in monkeys, and by Kuczynski (3) in mice. More recently Duval and Hibberd (4) have produced changes of acute nephritis by means of a streptococcal lysate prepared *in vivo* in the peritoneum of immunised animals. Rich, Bumstead and Frobisher (5) were the first to show that nephritis could be produced by means of a toxin present in the filtrate from young growing cultures. For this work they used streptococcus viridans. None of the animals injected were allowed to survive longer than one day and the only

[1] Received for publication Dec. 1, 1930.

[2] This paper is in the course of preparation.

lesion observed was haemorrhage into the glomerular capsules and into the tubules. As far as we have been able to ascertain, no experimental work on nephritis has been done with the pneumococcus.

PNEUMOCOCCAL AUTOLYSATE

The method used in the production of the pneumococcal autolysate has been, but for slight and unessential modifications, that described by Parker (6) in 1928. In every case the complete sterility of the autolysate was demonstrated by culture. In order to ascertain whether the toxic properties of this autolysate might be due to the action of the disintegrating bacteria upon the contents of the broth in which autolysis occurs rather than to an actual endotoxin produced by their disintegration, experiments have been carried out in which the pneumococci have been allowed to autolyse in normal saline. Autolysate prepared in this way causes a well-marked skin reaction in guinea-pigs and produces changes in the kidney resembling in every way those obtained after injection of broth autolysate. These experiments are as yet incomplete and protocols of the results are not included in the paper.

In these experiments forty-four rabbits have been used. All the injections have been made intravenously, but the quantity of autolysate injected dose by dose and the total number of doses given has varied. The appendix shows these variations in detail, but it may be said briefly that the quantities varied from 0.3 cc. to 2.4 cc. of pure autolysate and that the number of injections varied from 1 to 9, injections in every case being made daily. Of these 44 rabbits, 41, or 93.1 per cent, showed definite changes in the kidneys, in the glomeruli or the tubules, or in both (Table A). It has been found in general that the degree of these changes varies directly with the quantities of autolysate injected, and inversely with the size of the rabbits used, although, apart from these generalisations, marked individual differences were found among the rabbits.

Macroscopically, a few of the kidneys showed no changes which could be recognised as abnormal. In the majority of instances, however, they were swollen, definitely oedematous and of either a red or a pale yellow color (Figs. I and II). In a certain number minute petechial haemorrhages could be seen as small bright red pin-points on the surface or within the cortex, while in the large swollen yellow kidneys

TABLE A

SERIES	NUMBER OF RABBITS	EVIDENCE OF INJURY TO GLOMERULAR CAPILLARY TUFT				EVIDENCE OF INJURY TO RENAL EPITHELIUM		OEDEMA OR ASCITES
		Hyaline or fibrin thrombi	Blood in tubules	Fibrin casts in glomerular capsules or tubules	Cresent formation	Colloid drops in cells	Necrosis of epithelium	
A[1]	3	Negative	Negative	1 positive	Negative	Negative	Negative	Negative
A[2]	3	Negative	2 positive	1 positive	Negative	Negative	Negative	Negative
F	6	Negative	Negative	6 positive	Negative	Negative	6 positive	3 positive
G	8	1 positive	2 positive	8 positive	Negative	5 positive	6 positive	4 positive
I	12	2 positive	3 positive	12 positive	Negative	3 positive	12 positive	5 positive
J	6	Negative	3 positive	6 positive	Negative	Negative	6 positive	1 positive
K	6	3 positive	5 positive	6 positive	3 positive	Negative	5 positive	5 positive
Total.....	44	6 positive	15 positive	40 positive	3 positive	8 positive	35 positive	18 positive
Percentage positive.....		13.6%	34%	90.9%	6.1%	18.1%	79.5%	40.9%

The numerals in the columns of the chart indicate the number of rabbits in each series in which the injury designated was found in the kidneys.

there were very numerous minute yellow flecks all over the surface and these appeared as yellow lines in the cortex. Such yellow lines were found to correspond, in the microscopical preparations, either with dilated tubules containing casts or with actual necrotic tubules.

In the majority of cases paraffin sections were prepared from both kidneys, the tissues having been fixed in Zenker-formol; in the remainder, sections were made of only one kidney. These sections were stained with haematoxylin and eosin and with a modification of

FIG. I

FIG. II

FIG. I. Kidney of Rabbit F-9. Autolysate prepared in broth. Kidney is swollen and pale with many minute yellow flecks.

FIG. II. Kidney of Rabbit K-1. Autolysate prepared in broth. Kidney swollen. Numerous yellow and gray flecks and many small haemorrhages.

Weigert's fibrin stain. Frozen sections stained for fat with Scharlach R and counter-stained with Weigert's haematoxylin were also examined.

In eight cases complete autopsies were performed and microscopical sections studied from most of the organs (See Table B). Besides these eight, single sections of lung, liver, heart and intestine were examined in many instances.

Microscopically, the early lesions produced were haemorrhage from

TABLE B

NUMBER	HEART	VOLUNTARY MUSCLE	LUNG	LIVER	SPLEEN	ADRENAL	LARGE INTESTINE	SMALL INTESTINE	STOMACH	BRAIN	CORD
J-4	Small area of fresh haemorrhage		0	0	0	0	0	0			
J-5	Vacuolated fibres. Few small haemorrhages. Some mononuclear infiltration		0		0		Slight oedema				
J-6	0		0	0	0		0			0	0
K-2	Very few hyaline fibres		0	0	0	0	Marked oedema	Marked oedema	0		
K-3	0		Slight fresh haemorrhage	0	0		Oedema	Oedema	0		
K-4	Fibres show vacuoles in cytoplasm and nuclei. Some fibres hyaline	Marked vacuolisation and Zenker's necrosis	0	0	0	0	Marked oedema. Haemorrhage in submucosa	Marked oedema	Oedema of submucosa	0	0
K-5	Vacuolisation. Hyaline fibres	Vacuolisation. Zenker's necrosis	Fresh haemorrhage	0	0	0	Oedema	Oedema	0	0	0
K-6	0	Zenker's necrosis	0	0	0	0	Slight oedema	Slight oedema			

Where no entry is made the tissues were not examined.
0-negative.

the glomerular tufts with blood in the capsule and in the tubules, and the accumulation of colloid droplets within the tubular epithelium.[3]

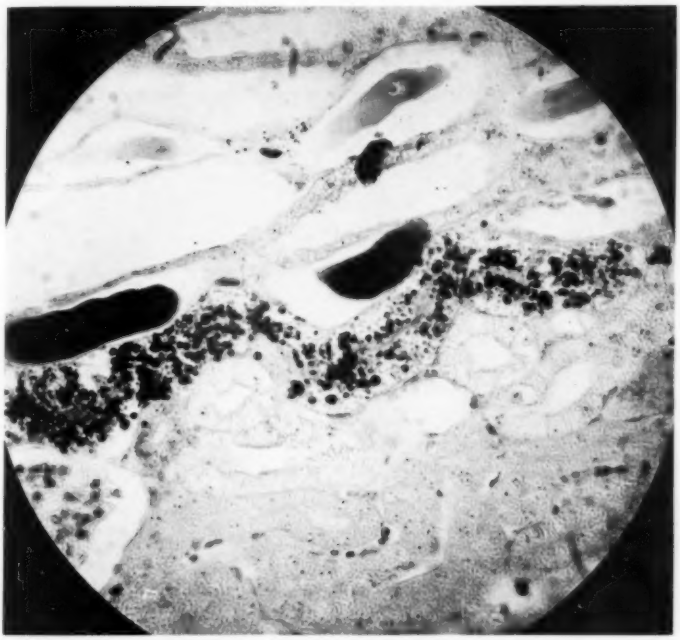

FIG. III. Rabbit G-3. Autolysate prepared in broth. Cells of the tubular epithelium contain colloid droplets. Weigert's fibrin stain.

[3] In connection with these colloid droplets it should be pointed out that two entirely distinct forms occur. In the one the droplets are numerous, small, and do not stain very deeply. This type is not seen in human kidneys; it is found frequently in rabbits injected with autolysate and also in rabbits infected with pneumococci, but it can be demonstrated in the rabbits used as a control which have been injected with the broth alone. The other type of droplet is large, discrete and deeply staining and is found in much smaller numbers (Fig. III). It is found in some of the rabbits given the ordinary autolysate besides that in which autolysis has occurred in normal saline, and resembles the droplets seen in human nephritis or tubular damage and has not been found in the controls. Neither form of droplet is found in the normal control rabbit taken from stock and killed immediately.

At this stage of renal damage or a little later, the capillary loops in the glomeruli were found to contain many fresh fibrin or older hyaline thrombi.

The further changes were all so interwoven that it has been impossible to be certain which stages precede and which follow. The

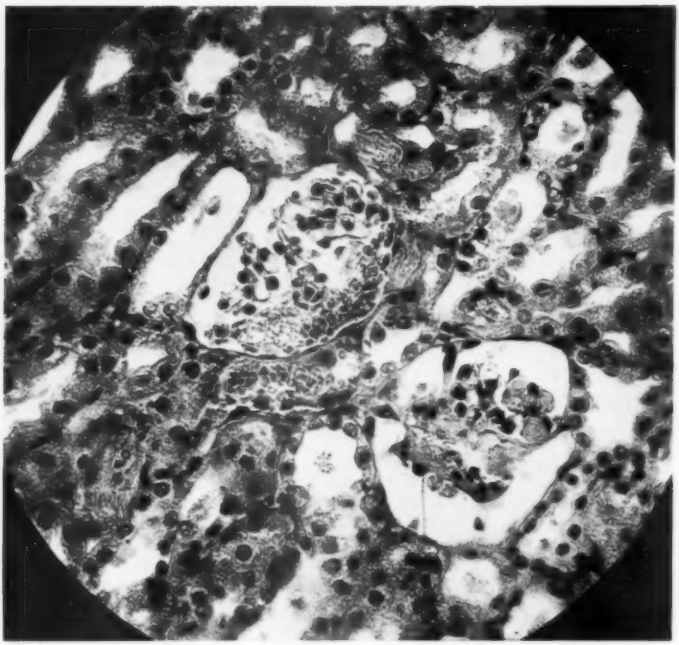

FIG. IV. Rabbit C-4. Autolysate prepared in broth. In the upper glomerulus the capsule is distended and contains red blood cells. The tubule below contains a few red blood cells. In the lower glomerulus there are two adhesions and the capillary loops on the right contain hyaline thrombi.

glomerular capsules became dilated. They were empty or contained coagulated fluid, fibrin, and clumps of red blood cells (Fig. IV). Some showed definite adhesions of the capillary loops to the wall of the capsule. In a certain number minute but distinct colloid droplets could be demonstrated in the epithelium covering the glomerular tuft, and

fat stains revealed a little fat in droplets within the tuft. At the same time marked changes were found in the tubules. In many places the epithelium was necrotic and this would seem to be a further stage of the accumulation of colloid droplets. The tubules became dilated and lined with a regenerating and flattened epithelium. They con-

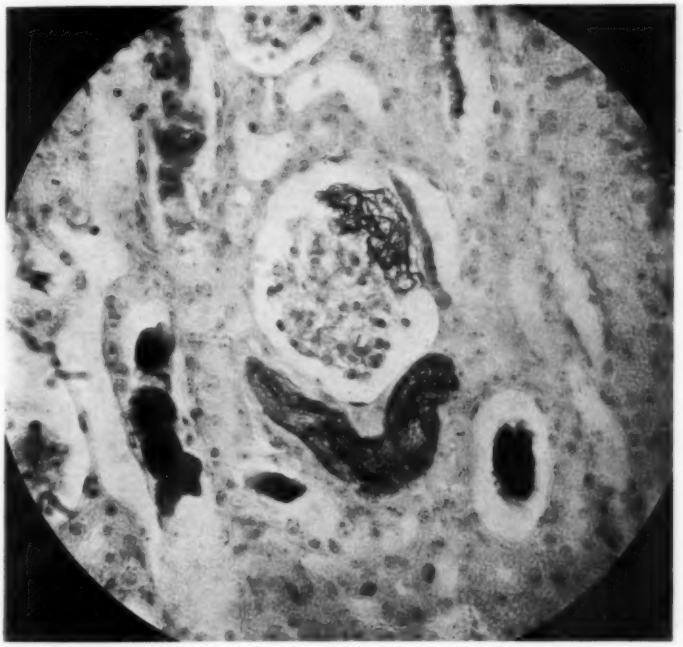

FIG. V. Rabbit G-4. The glomerular capsule and the adjacent tubule contain fibrin casts. Other tubules contain hyaline casts. Weigert's fibrin stain.

tained casts which were of many kinds, fresh fibrin, red blood cells, hyaline or granular material, and, in a few instances, masses of leucocytes (Figs. V, VI). Fat stains, as a rule, did not show much fat within the tubular epithelium, but fat droplets could be seen lying between the tubules in the interstitial tissue, in the walls and in the lumina of the capillaries and medium-sized vessels. The walls of some of the smaller arteries or pre-arterioles appeared hyaline.

The final stages observed in this series were as follow. In the glomerular spaces in which the masses of fibrin and cells remain, the cells of Bowman's capsule commenced to increase in size and showed every evidence of new activity (Fig. VII). This we believe to be the first stage in the formation of a crescent by the invasion of the fibrin masses

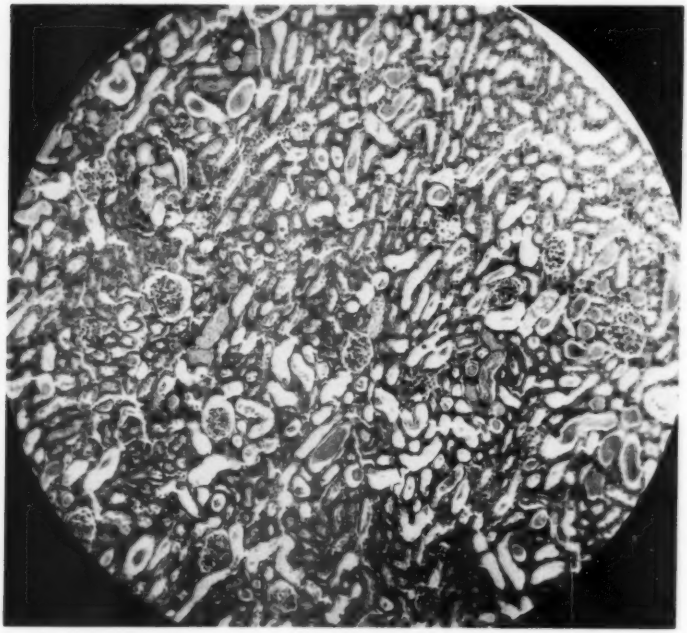

FIG. VI. Rabbit F-4. Autolysate prepared in broth. The dilatation of the tubules and glomerular capsules is seen. Some of the tubules show necrotic epithelium. There are many hyaline and granular casts.

with such cells, and indeed such an invasion with the formation of what were unmistakably early crescents was seen in a few instances. The terminal stages observed in the tubules consisted most frequently in marked dilatation with strikingly flat lining epithelium. In a certain number of cases, however, the necrotic tubules had become definitely calcified.

The sections stained with Weigert's fibrin stain were carefully examined for bacteria, but none were seen in any case.

Among the 44 rabbits there were 18, or 40.9 per cent, which showed quite marked generalised oedema or ascites or both. The oedema was most extensive in the intestine, especially in the appendix, in the

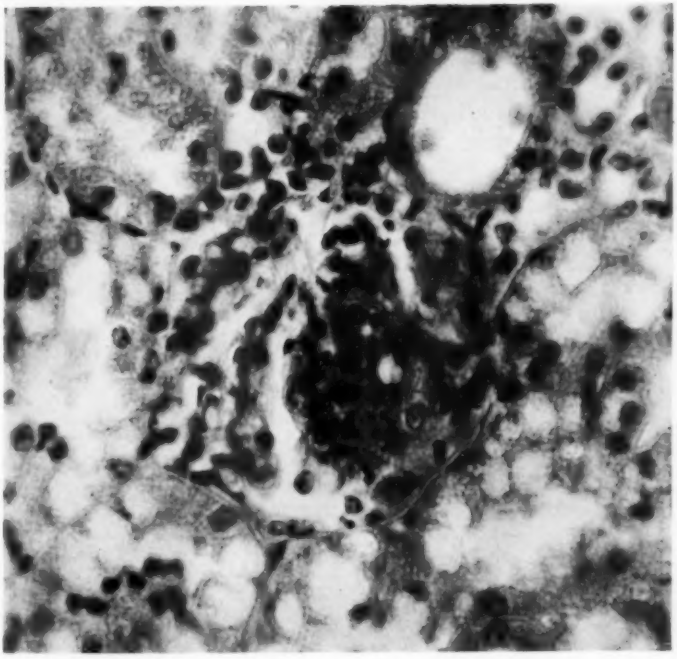

FIG. VII. Rabbit K-4. Autolysate prepared in broth. Regenerating glomerular and capsular epithelium forms a mass occupying half of the capsular space. There are at least two mitotic figures shown.

voluntary muscles, and in the subcutaneous tissue. It occurred most frequently in the rabbits which had received the largest quantity of autolysate.

The other organs, apart from the lungs, showed no changes that could be recognised in the gross except for spontaneous parasitic infections, in particular, coccidiosis.

Microscopically, the liver, spleen, adrenal, stomach, brain and spinal cord were uniformly normal. The lungs showed in a few instances fresh haemorrhage, while in some others the alveoli contained clear fluid. In no instance could any lesion be observed in the alveolar walls. There was no pneumonia. The intestine from the rabbits with generalised oedema showed marked infiltration of fluid into the submucosa which was widely spread apart. Occasionally there were small fresh haemorrhages. In a few instances the oedematous submucosa was infiltrated with leucocytes, but this seemed to be associated with a secondary parasitic invasion of the oedematous wall.

Apart from the renal lesions and the oedema, the only other striking abnormality observed was in the muscle, both cardiac and voluntary. In the heart the change was found most frequently in the wall of the left ventricle and consisted in the presence of certain groups of hyaline fibres in which all structure was lost, together with other fibres which showed vacuoles both in the cytoplasm and within the nuclei. In the voluntary muscles the changes were even more marked. In association with the oedema recognised in the gross, the muscle fibres were found filled and distorted with vacuoles in a surprising manner. Quite distinct from this, however, was the presence of numerous swollen, hyaline and disintegrating fibres. Some of these were surrounded and even invaded by polymorphonuclear leucocytes, and the whole picture was identical with the Zenker's necrosis of voluntary muscle described by Forbus (7) in a certain percentage of pneumonias caused by the pneumococcus, the streptococcus, or B. influenzae,—predominantly by the first. We are at present engaged in a study of these changes in the muscle and in a comparison of the lesions obtained in rabbits with those observed in human autopsy material.

The changes in the blood and urine have not as yet been followed. It is clear that such changes will be of value in completing the resemblance between the condition produced in rabbits and that seen in man. As a result of the experimental procedure the greater number of rabbits became definitely ill. They sat more or less immobile in one corner of the cage, refusing their food and showing a bedraggled appearance of the fur. They became rapidly emaciated and the lethargy became more marked. In a certain number, and these were usually those in which the intestinal oedema was marked, there was

pronounced diarrhoea. As a final stage the rabbits usually went into coma with shallow respirations, and death followed in a short while. In a few the coma was accompanied by marked clonic convulsions affecting the limbs and the vertebral muscles in particular, but occasionally becoming generalised.

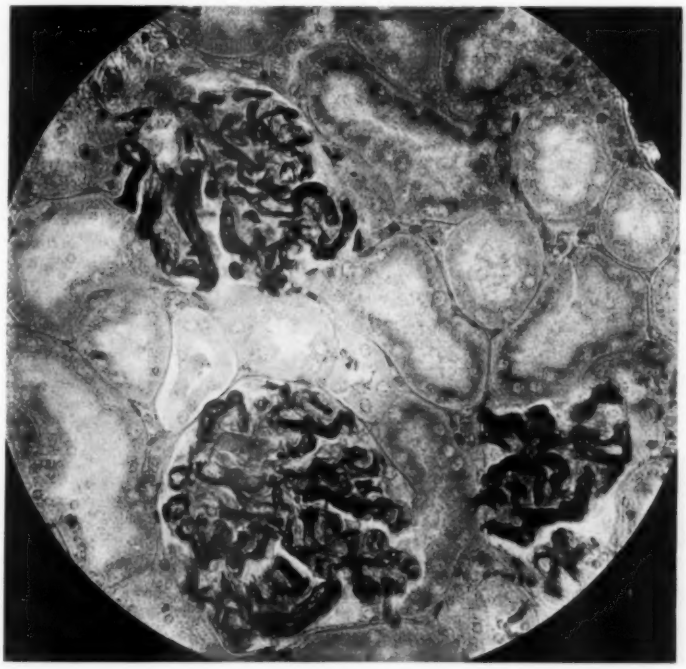

FIG. VIII. Rabbit A-4. Intradermal infection, Pneumococcus Type I. The capillaries of the glomerular tufts are filled with fibrin thrombi. Weigert's fibrin stain.

Twenty-four control rabbits were injected with the same veal peptone broth as was used in the preparation of the autolysate. The doses given were either 1 or 2 cc. and the rabbits were injected daily for six days or longer. All of these animals showed moderate numbers of the smaller type of colloid granules. Apart from this change

none of the twenty-four showed any lesions in the kidneys with the exception of H_7, in which a little fat was present as fine droplets in some of the convoluted tubules.

PNEUMOCOCCAL INFECTION

A small series of rabbits has been infected with pneumococci injected, for the most part, intradermally in doses ranging from 0.2 cc. to 0.5 cc. of a moderately heavy one-day-old broth culture. Types I, II and III have been used and but little attention has been paid, up to the present, to the virulence of the particular strains used. It has been found that Type I produced the most marked effect not only in regard to the local skin reaction and the systemic upset but also in the kidneys. Of 15 rabbits inoculated with Type I, eleven received what seemed to be a virulent strain, and of these 6, or 54.5 per cent, showed early changes of nephritis as represented by haemorrhage from the glomeruli into the tubules in all, by the presence of colloid granules in the epithelium of the tubules in 3, and by the occlusion of the glomerular capillaries with fibrin thrombi in 2 (Fig. VIII).

Eight rabbits were injected with Type II, an avirulent strain. Of these, one showed colloid granules in the convoluted tubules but nothing else.

Eleven were given Type III and all of these showed definite skin reactions at the site of infection. Of these eleven, four, or 36.4 per cent, showed colloid granules in the tubular epithelium.

In every case the Weigert fibrin stains showed the presence of pneumococci in the capillaries of the kidneys, demonstrating that a general septicaemia had occurred. These organisms were always single or in groups of two or three; in no instance were there any clumps which might conceivably be thought to be blocking the capillaries.

NEPHRITIS IN MAN ASSOCIATED WITH PNEUMOCOCCUS INFECTION

As has already been said, we are at present engaged in an investigation of the material in our autopsy records of patients dying from a pneumococcus infection whether of the meninges, the middle ears or sinuses, the lungs and pleurae, the endocardium and pericardium, or the peritoneum. Since this work is not as yet completed, definite figures cannot be given, but it may be said that from 40 to 50 per

cent show changes very similar to those described in our rabbits. These changes have included blood and fibrin in the glomerular capsules, and in the tubules, fibrin and hyaline thrombi, colloid droplets in the tubular epithelium, dilatation of the tubules with hyaline and granular casts, and, in certain instances, necrosis and calcification of the epithelium of the tubules. There are also glomerular adhesions and crescents.

SUMMARY

Characteristic acute and subacute nephritis has been produced in rabbits by means of an autolysate prepared from Type I Pneumococcus and also by means of intradermal infection with virulent strains of pneumococci. The toxin affects the glomerular capillaries, as evidenced by the hyaline and fibrin thrombi, and the blood and fibrin in the tubules, and produces injury and necrosis in the epithelium of the tubules and glomeruli. In a certain number of cases the damage to the kidneys has been associated with marked oedema of the tissues and with ascites.

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APPENDIX

Series 1

Rabbit 1. Given 1 dose (1.5 cc.) of autolysate. Killed 27½ hours later.
Rabbit 2. Died 3 hours after injection of first dose of autolysate (0.7 cc.).
Rabbit 3. Given 1 dose (0.25 cc.) of autolysate. Killed 26 hours later.

Series 2

Rabbit 1. Given 9 small daily doses. Total dosage 3.85 cc. Killed 30½ hours after last dose.
Rabbit 2. Given 6 small daily doses. Total dosage 2.57 cc. Died 12 hours after last dose.
Rabbit 3. Given 9 small daily doses. Total dosage 4.07 cc. Killed 30½ hours after last dose.

Series F

Rabbit 1. Given 4 daily doses of 1 cc. each. Killed 12 hours after last dose.
Rabbit 2. Given 5 daily doses of 1 cc. each. Died 5 hours after last dose.
Rabbit 3. Given 4 daily doses of 1 cc. each. Died 5 hours after last dose.
Rabbit 4. Given 4 daily doses of 1 cc. each, and 1 dose of 0.5 cc. Died 5 hours after last dose.
Rabbit 5. Given 4 daily doses of 1 cc. each. Killed 24 hours after last dose.
Rabbit 6. Given 4 daily doses of 1 cc. each. Died 20 hours after last dose.

Series G

Rabbit 1. Given 1 dose of 1 cc. Died 20 hours later.
Rabbit 2. Given 2 daily doses of 1 cc. each. Died 10 hours after last dose.
Rabbit 3. Given 3 daily doses of 1 cc. each. Killed 18 hours after last dose.
Rabbit 4. Given 5 daily doses of 1 cc. each. Killed 4 hours after last dose.
Rabbit 5. Given 5 daily doses of 1 cc. each. Died 20 hours after last dose.
Rabbit 6. Given 2 daily doses of 1 cc. each. Died 20 hours after last dose.
Rabbit 7. Given 2 daily doses of 2 and 2.4 cc., respectively. Died 24 hours after last dose.
Rabbit 8. Given 3 daily doses, 2.4, 1.75, and 1.2 cc. Killed 48 hours after last dose.

Series I

Rabbit 1. Given 1 dose of 2 cc. and 1 dose of 1 cc. Killed 8¾ hours after last dose.
Rabbit 2. Given 1 dose of 2 cc. and 1 dose of 1 cc. Died 2½ hours after last dose.
Rabbit 3. Given 1 dose of 2 cc. and 1 dose of 1 cc. Killed 8 hours after last dose.
Rabbit 4. Given 1 dose of 2 cc. Died 24 hours later.
Rabbit 5. Given 1 dose of 2 cc. Died 24 hours later.
Rabbit 6. Given 1 dose of 2 cc. and 1 dose of 1 cc. Killed 9 hours after last dose.

Rabbit 7. Given 3 daily doses, 1 cc., 1 cc., and 0.7 cc. Died 2 hours after last dose.
Rabbit 8. Given 1 dose of 1 cc. Killed 20 hours after last dose.
Rabbit 9. Given 2 doses, 1 cc. and 0.7 cc., respectively. Killed 12 hours after last dose.
Rabbit 10. Given 1 dose of 1 cc. Died 20 hours later.
Rabbit 11. Given 1 dose of 1 cc. Killed 20 hours later.
Rabbit 12. Given 4 doses, 1 cc., 0.7 cc., 1 cc. and 2 cc. Died 1 hour after last dose.

Series J

Rabbit 1. Given 4 daily doses of 1 cc. each. Died 20 hours after last dose.
Rabbit 2. Given 4 daily doses of 1 cc. Died 20 hours after last dose.
Rabbit 3. Given 5 daily doses of 1 cc. each. Died 6 hours after last dose.
Rabbit 4. Given 2 daily doses of 2 cc. each and 5 daily doses of 1 cc. each. Died 6 hours after last dose.
Rabbit 5. Given 1 dose of 2 cc. and 6 daily doses of 1 cc. each. Killed 24 hours after last dose.
Rabbit 6. Given 3 daily doses, 1.5 cc., 1 cc., and 0.7 cc. Killed 28 hours after last dose.

Series K

Rabbit 1. Given 3 daily doses of 1 cc. each. Killed 5 hours after last dose.
Rabbit 2. Given 4 daily doses of 1 cc. each. Died 20 hours after last dose.
Rabbit 3. Given 5 daily doses of 1 cc. each. Died 9 hours after last dose.
Rabbit 4. Given 6 daily doses of 1 cc. each and 1 dose of 2 cc. Killed 7 hours after last dose.
Rabbit 5. Given 6 daily doses of 1 cc. each and 1 dose of 2 cc. Killed 14 hours after last dose.
Rabbit 6. Given 6 daily doses of 1 cc. each and 1 dose of 2 cc. Killed 24 hours after last dose.

STUDIES IN EXPERIMENTAL SYPHILIS

IX. The Distribution of the Resistant State in "Immune Rabbits"[1,2]

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INTRODUCTION

The state of resistance which develops in the rabbit during the course of experimental syphilis appears to be widely distributed amongst different tissue groups but not to the same degree. This seems to be particularly true in the case of the eye. The extensive studies of Brown and Pearce (1) have yielded a considerable body of information upon the distribution of the resistant state amongst the various tissues during the course of the first infection, but the problem, in so far as it applies to the course of events following a second infection, has not received the same degree of attention, although Uhlenhuth and Mulzer (2), Tomasczewski (3), Frei (4) and Mulzer and Nothhaas (5) have all reported experiments having a bearing upon this question. Not only does it seem desirable to ascertain as far as possible the precise distribution of the syphilis-resistant state but it is important to find out if this distribution is affected by the manner in which the infection is produced. The experiments reported in this communication were designed to throw light upon this particular question.

Most of the recent reinoculation experiments in syphilitic rabbits have been carried out with the intratesticular or subscrotal method of inoculation, both for the primary and for subsequent inoculations. Much less attention has been given to other methods, such as intracutaneous, subcutaneous and intravenous injection, and deposition of the virus upon the surface of a granulating wound (6). For that reason we have concerned ourselves, in these experiments, with these

[1] Aided by a grant from the Committee on Research in Syphilis.

[2] Submitted for publication November 14, 1930.

less used methods of inoculation, and have sought to determine whether the manner of inoculation has any influence upon the development of resistance to a second syphilitic infection.

Stated in more specific terms, the experiments herein reported were designed to afford answers to the following questions:

1. Does a first infection with syphilis in the rabbit, brought about by a method of inoculation which involves the skin (intracutaneous injection, deposition of virus upon a granulating wound), protect the rabbit against syphilitic disease phenomena when homologous syphilitic virus is subsequently inoculated intratesticularly, subcutaneously, intracutaneously or intravenously?

2. Does a first infection with syphilis brought about by intratesticular inoculation protect the rabbit against syphilitic disease phenomena when homologous virus is inoculated intracutaneously?

3. Does a first infection with syphilis brought about by intravenous inoculation protect the rabbit against a second infection as well as against disease phenomena when homologous virus is inoculated intravenously, subcutaneously or intracutaneously?

4. Does a first infection with syphilis brought about by subcutaneous injection protect the rabbit against disease phenomena when homologous virus is inoculated intratesticularly, intracutaneously or intravenously?

In regard to the last question it should be stated that it was our hope that by inoculating subcutaneously it would be possible to produce in the rabbit infection without any local lesion at the portal of entry, and thus have an opportunity for testing the resistance developing after a symptomless infection, as has been done by Manteufel and Richter (7) Prigge and Rothermundt (8) and by Kigasawa (9). In practice, however, it was not found possible to accomplish this result, since in all the animals inoculated subcutaneously a lesion developed in the subcutaneous tissue where the inoculum was deposited, or more frequently in the skin itself where it was penetrated by the needle. The lesions which did develop in the subcutaneous tissue quickly involved the overlying skin, so that no lesion strictly limited to the subcutaneous tissue was obtained. Accordingly, all the animals in the experiment designed to afford an answer to the last question actually fell in the category of those inoculated in such a

manner as to yield a lesion involving the skin, and for that reason should be considered along with those inoculated intracutaneously or by deposition of the virus upon a granulating wound. They should be regarded, therefore, as affording an answer to the first question.

In formulating the experiments designed to answer the questions outlined above, it was necessary to confine our observations to what happened at the site of the second inoculation, and to disregard the ultimate fate of the virus used for reinoculation, save in those instances where the second inoculation was made intravenously. It would have been desirable to have sought to determine, in each instance, what finally happened to the virus used for inoculation, as disclosed by subsequent lymph node transfer from the reinoculated animals, but lack of sufficient space for the large number of animals which would have been needed for the purpose prevented the carrying out of this project. It was, however, absolutely necessary to carry out subsequent lymph node transfers in the case of those animals inoculated intravenously and this was done. The experiments therefore afford an idea of the distribution of the syphilis-resistant state in the rabbit's body in so far as the development of a local lesion at the site of reinoculation is concerned, also the occurrence of clinically detectable metastatic lesions, but to a limited degree only are they concerned with the problem of absolute protection or complete immunity.

EXPERIMENTAL

The experiments herein reported were begun February 1927, and extended over a period of more than 2 years. Certain general conditions obtained in all the experiments and may be cited at this point to avoid repetition.

The virus used for both first and subsequent inoculations was the well-known Nichols strain. Treatment, where carried out, consisted of 6 intravenous injections of arsphenamine, 20 mgm. per kilogram of body-weight, at intervals of a week between each injection. Where the Wassermann test was made, it was carried out according to the technic routinely employed in the Wassermann laboratory of the Johns Hopkins Hospital, and previously described (10). Animals to which emulsions of lymph nodes were transferred, were observed for a period of at least 90 days and in most instances for a longer period

before being reported as negative. Only the test animals surviving 90 days after the second inoculation are included in the protocols. Some of the test animals died before the experiments were completed.

Experiment 1. This experiment corresponds to Question 1, mentioned above. Altogether, 33 rabbits were inoculated with syphilitic virus in such a manner as to produce a primary lesion in-

TABLE I

Result of reinoculation of syphilitic rabbits by intracutaneous inoculation

RABBIT NUMBER	SEX	FIRST INFECTION			SECOND INFECTION—INTRACUTANEOUS INOCULATION			CONTROLS—INTRACUTANEOUS INOCULATION		
		Method	Local lesion	General lesions	Day after first infection	Local lesion	General lesions	Rabbit number	Local lesion	General lesions
1975	F.	Subcutaneous	+	0	288	0	0	2317	+	0
1976	F.	Subcutaneous	+	0	288	0	0	2319	+	0
1978	F.	Subcutaneous	+	0	288	0	0	2320	+	0
1979	F.	Subcutaneous	+	0	288	0	0	2427	+	0
2319	F.	Intracutaneous	+	0	128	0	0	2433	+	0
2320	F.	Intracutaneous	+	0	128	0	0	2437	+	+ (T.)
2321	F.	Intracutaneous	+	+ (T.S.E.)	128	0	0	2439	+	0
2322	F.	Intracutaneous	+	0	128	0	0	2442	+	0
2324	F.	Intracutaneous	+	0	128	0	0	2443	+	+ (T.E.)
2325	F.	Intracutaneous	+	0	128	0	0	2444	+	0
2326	F.	Intracutaneous	+	0	128	0	0	2445	+	+ (T.)
2330	F.	Intracutaneous	+	+ (B.)	128	0	0	2447	+	+ (B.)
2331	F.	Intracutaneous	+	0	128	0	0	2450	+	0
2332	F.	Intracutaneous	+	0	128	0	0	2451	+	0
2333	F.	Intracutaneous	+	0	128	0	0			

T., metastatic testicular lesion; B., periosteal—bone lesion; E., eye lesion; S., skin lesion.

volving the skin of the back. Of these, 11 were inoculated intracutaneously, 13 subcutaneously, and 9 by the deposition of virus on the surface of a granulating wound. In all of them characteristic syphilitic lesions developed at the site of inoculation. Some were treated, others not. Where treatment was carried out, it was begun late in the course of the infection, that is, 187 days after inoculation. Second inoculations with homologous virus were made intracutaneously,

subcutaneously, intratesticularly and intravenously. Normal control rabbits were of course inoculated in a similar manner. For the sake of convenience in presenting the data, these animals have been arranged in groups according to the method of the second inoculation. The results of the experiment are shown in Tables I, II, III and IV.

Table I shows that in 4 rabbits inoculated subcutaneously there developed a local lesion which ultimately involved the skin. These same rabbits inoculated intracutaneously 288 days later with homologous virus showed no lesions at the site of inoculation and no metastatic lesions during the period of observation. In all 3 controls lesions appeared at the site of the intracutaneous inoculation. It is also seen from the table that 11 rabbits inoculated intracutaneously

TABLE II

Result of reinoculation of syphilitic rabbits by subcutaneous inoculation

RABBIT NUMBER	SEX	FIRST INFECTION—WOUND INOCULATION			SECOND INFECTION—SUBCUTANEOUS INOCULATION 344TH DAY			CONTROLS—SUBCUTANEOUS INOCULATION			
		Local lesion	General lesions	WaR.	Local lesion	General lesions	WaR.	Rabbit number	Local lesion	General lesions	WaR.
1673*	F.	+	+ (S.)	+	0	0	0	1974	+	0	0
1675*	F.	+	0	+	0	0	0	1975	+	0	0
1677*	F.	+	+ (B.)	+	0	0	0	1976	+	0	+

* Treated with arsphenamine, 6 × 20 mgm./kgm. on 182nd day.

all showed lesions at the site of inoculation and in 2 of them metastatic lesions were observed. All of these, upon a second inoculation 128 days later with homologous virus, failed to develop any lesions at the site of inoculation and gave no sign of metastatic lesions, while in all of the 11 control rabbits there developed characteristic lesions at the site of inoculation, and in 4 of these metastatic lesions were encountered during the period of observation.

Table II shows that in each of 3 rabbits inoculated by the granulating wound method, a lesion developed at the site of inoculation, and in 2 of them metastatic lesions were observed. All 3 were inoculated with homologous virus 344 days later by subcutaneous injection. At that time no clinical evidence of active syphilitic disease was manifest. In none of these animals did a lesion develop although the 3 controls similarly inoculated exhibited characteristic lesions.

Table III shows that in each of 2 rabbits inoculated by the granulating wound method, and in each of 5 inoculated by subcutaneous injection a lesion developed at the site of inoculation and metastatic lesions were observed in 2. All of them were inoculated intratesticularly with homologous virus 344 days after the first inoculation, at a time when the animals were free from lesions attributable to the first infection. In none of them was a lesion observable at any time after the reinoculation. The Wassermann reaction remained negative at all times. In all of the controls inoculated intratesticularly typical

TABLE III

Reinoculation of syphilitic rabbits by intratesticular injection—first inoculation upon a granulating wound or by subcutaneous injection

RABBIT NUMBER	SEX	METHOD	FIRST INFECTION			SECOND INFECTION—344TH DAY OF DISEASE, INTRATESTICULAR INOCULATION			CONTROLS—INTRATESTICULAR INOCULATION			
			Local lesion	General lesions	WaR.	Local lesion	General lesions	WaR.	Rabbit number	Local lesion	General lesions	WaR.
1499*	M.	Wound	+	+ (E.)	+	0	0	0	1943	+	+ (T.B.)	+
1549*	M.	Wound	+	0	+	0	0	0	1944	+	+ (T.E.)	+
1641*	M.	Subcutaneous	+	0	+	0	0	0	1945	+	+ (E.)	+
1648*	M.	Subcutaneous	+	+ (T.B.)	+	0	0	0	1946	+	+ (E.)	+
1650*	M.	Subcutaneous	+	0	+	0	0	0	1947	+	0	+
1654*	M.	Subcutaneous	+	0	+	0	0	0				
1656*	M.	Subcutaneous	+	0	+	0	0	0				

* Treated with arsphenamine, 6 × 20 mgm./kgm. on 182nd day.

lesions of syphilitic orchitis developed within the usual incubation period, together with the occurrence of a positive Wassermann reaction and a high incidence of metastatic lesions.

Table IV shows the results of intravenous reinoculation of 8 syphilitic rabbits that had been previously inoculated by either the granulating wound method or by subcutaneous injection. In all 8 of these animals a characteristic primary syphilitic lesion developed at the site of inoculation following the first injection and in 6 of them metastatic lesions were observed. All 8 were treated 187 days after the first inoculation, at a time when they were exhibiting no lesions. A popli-

TABLE IV

Result of intravenous reinoculation of treated syphilitic rabbits

RABBIT	SEX	METHOD	FIRST INFECTION			TREATMENT BEGUN 187TH DAY		SECOND INFECTION—INTRAVENOUS INOCULATION			CONTROLS—INTRAVENOUS INOCULATION		
						First node transfer		346th day of disease					
			Local lesion	General lesions	WaR.	Days after treatment	Result	General lesions	WaR.	Second node transfer	Rabbit number	General lesions	WaR
1864	M.	Wound	+	+ (T.)	+	107	0 0	0	0	+ +	2308	+ (T.)	+
1865	M.	Wound	+	+ (B.E.)	+	107	0 0	0	0	D 0	2309	+ (T.)	+
1869	M.	Wound	+	+ (B.S.E.T.)	+	107	0 0	0	0	+ +	2310	+ (T.B.S.)	+
1870	M.	Wound	+	0	+	107	0 0	0	0	0 0	2311	+ (T.S.)	+
1878	M.	Subcutaneous	+	+ (T.S.B.)	+	107	0 0	0	0	D 0			
1879	M.	Subcutaneous	+	+ (T.)	+	110	0 0	0	0	+ +			
1880	M.	Subcutaneous	+	0	+	110	D 0	0	0	0 0			
1887	M.	Subcutaneous	+	+ (T. E.)	+	110	D 0	0	0	0 0			
1901	M.	Subcutaneous	+	0	+	110	D 0	Not reinoculated		0 0	Treatment controls		
1904	M.	Subcutaneous	+	0	0	110	0 0	Not reinoculated		0 0			
1922	M.	Wound	+	+ (B.T.E.)	+	107	D D	Not reinoculated		0 0			

First node transfer carried out 294th to 298th day of disease.

Second node transfer carried out 444th day of disease, 95th day after second inoculation and 219th day after last treatment.

teal lymph node was removed from each animal 107 to 110 days after treatment had ceased. After emulsification in salt solution it was inoculated intratesticularly into each of 2 normal rabbits which were kept under observation for 90 days. All 8 node transfers proved negative. The test animals were then reinoculated intravenously with homologous virus, 346 days after the first inoculation. No syphilitic lesions were observed in any of these animals and the Wassermann reaction remained negative although the four control rabbits inoculated intravenously at the same time all exhibited lesions. A second lymph node transfer from the test animals, performed 95 days after reinoculation and 218 days after the last injection of arsphenamine gave positive results in 3 of the 8 test animals. The 3 treated animals which were not reinoculated but were used as treatment controls all gave negative node transfers, indicating that treatment had sufficed to eliminate the first infection. It would appear that at least 3 of the 8 test animals had been successfully reinoculated by intravenous injection although no lesions were observed at any time. The resistant state that develops in rabbits during the course of syphilis is apparently not proof against an intravenous injection of homologous virus, to judge from these experiments, although it is of sufficient magnitude to protect susceptible tissues against the occurrence of lesions, even after intravenous inoculation.

Experiment 2. This experiment was designed to afford an answer to the second question outlined above, namely, whether the resistant state which develops in syphilitic rabbits after intratesticular inoculation is imparted to the skin as determined by intracutaneous inoculation.

Six test animals, divided into 2 groups of 3 each, were available for this experiment. They were all inoculated intratesticularly and in all of them the lesions characteristic of syphilitic orchitis developed. The primary inoculations were carried out on different dates in the course of the two groups, but all 6 test animals were reinoculated on the same day with the same batch of virus. All were free of lesions at the time of the second inoculation. The results of this experiment are shown in Table V.

Table V shows that all 6 test animals inoculated intratesticularly showed characteristic local lesions and in 3 of them metastatic lesions

were encountered. When reinoculated intracutaneously with homologous virus 188 to 288 days after the first inoculation, the results were entirely negative. No lesions developed at the site of inoculation or

TABLE V

Result of reinoculation of syphilitic rabbits by intracutaneous injection after preliminary intratesticular inoculation

RABBIT NUMBER	FIRST INFECTION—INTRATESTICULAR INOCULATION		SECOND INFECTION—INTRACUTANEOUS INOCULATION			CONTROLS		
	Local lesion	General lesions	Day after first infection	Local lesion	General lesions	Rabbit number	Local lesion	General lesions
1943	+	+ (T.B.)	288	0	0	2317	+	0
1945	+	+ (E)	288	0	0	2319	+	0
1947	+	+ (T)	288	0	0	2320	+	0
2120	+	0	188	0	0	2321	+	+
2121	+	0	188	0	0	2322	+	0
2122	+	0	188	0	0			

TABLE VI

Result of reinoculation of syphilitic rabbits by subcutaneous injection after preliminary intravenous inoculation

RABBIT NUMBER	SEX	FIRST INFECTION—INTRAVENOUS INOCULATION		SECOND INFECTION—SUBCUTANEOUS INOCULATION, 344 DAYS AFTER FIRST INOCULATION			CONTROLS—SUBCUTANEOUS INOCULATION			WaR.
		General lesions	WaR.	Local lesion	General lesions	WaR.	Rabbit number	Local lesion	General lesions	
1632	F.	+ (S)	+	0	0	0	1977	+	0	+
1639	F.	0	±	0	0	0	1978	+	0	+
1680	F.	+ (S.B.)	+	0	0	0	1979	+	0	+
1681	F.	+ (B)	+	0	0	0	1980	+	0	+
1682	F.	+ (B)	+	0	0	0	1981	+	0	0
1684	F.	+ (B)	±	0?	0	0				
1685	F.	+ (B)	+	0	0	0				
1692	F.	+ (B)	±	0	0	0				

Rabbits 1632 to 1692 all treated with arsphenamine, 6 × 20 mgm./kgm.
Treatment begun 182nd day, completed 217th day.

elsewhere. The control animals all developed characteristic syphilitic lesions.

Experiment 3. This experiment was designed to afford an answer to Question 3 outlined above, namely, how will rabbits originally

rendered syphilitic by intravenous injection, react to a second infection with homologous virus inoculated (*a*) subcutaneously, (*b*) intracutaneously, or (*c*) intravenously? Fifteen rabbits were inoculated intravenously with syphilitic virus and the course of the disease followed clinically. Eight of these rabbits were subsequently inoculated subcutaneously, 5 intracutaneously, and 2 intravenously. Those reinoculated by intravenous injection were treated with arsphenamine, treatment being begun 187 days after inoculation, at a time when the animals were free from lesions. In these animals lymph

TABLE VII

Result of reinoculation of syphilitic rabbits by intracutaneous inoculation after preliminary intravenous inoculation

RABBIT NUMBER	SEX	FIRST INFECTION—INTRAVENOUS INOCULATION	SECOND INFECTION—INTRACUTANEOUS INOCULATION, 188 DAYS AFTER FIRST INOCULATION		CONTROLS—INTRACUTANEOUS INOCULATION		
		General lesions	Local lesion	General lesions	Rabbit number	Local lesion	General lesions
2010	F.	+ (S.B.)	0	0	2324	+	0
2013	F.	+ (S.)	0	0	2325	+	0
2049	F.	+ (S.B.)	0	0	2326	+	0
2051	F.	+ (S.B.)	0	0	2327	±	0
2052	F.	+ (S.)	0	0	2329	+	0
					2330	+	+ (B.)
					2331	+	+ (S.B.)
					2332	+	+ (S.)
					2333	+	0

node transfers were carried out both before and after the second inoculation. In the untreated animals there were no syphilitic lesions observable when the second inoculation was performed. The results of the experiment are shown in Tables VI, VII and VIII.

From Table VI it is seen that 7 of the 8 female rabbits inoculated intravenously with virulent syphilitic virus exhibited generalised lesions involving either the skin or the bones or both, but for the most part the latter. This finding is in conformity with that of Uhlenhuth and Grossmann (11). All 8 animals were treated with arsphenamine, treatment continuing from the 182nd to the 217th day of the infection. On the 344th day they were reinoculated with homologous virus by

subcutaneous injection. In one of them a suspicious lesion developed but this lesion was never definitely proved to be syphilitic in character; the remaining 7 never exhibited any lesions and the Wassermann reaction was repeatedly negative.

Table VII shows that 5 female rabbits which were inoculated intravenously with syphilitic virus all acquired generalised lesions and, when reinoculated with homologous virus by intracutaneous infection 182 days later, failed to show any lesions at the site of reinoculation.

Table VIII shows the result of reinoculation, by the intravenous route, of two rabbits that had originally been inoculated in the same

TABLE VIII

Result of reinoculation of syphilitic rabbits by intravenous inoculation after preliminary intravenous inoculation

Rabbit number	Sex	First infection		Treatment—days of disease		First node transfer		Second inoculation			Second node—day	Transfer, result	
		General lesions	WaR.	Begun	Ended	Day	Result	Day	General lesions	WaR.			
1914	M.	+ (T.B.)	+	221	256	328	0	345	0	0	444	+	+
1918	M.	+ (B.T.E.)	+	221	256	335	0	345	0	0	444	0	0
1911	M.	+ (B.S.T.E.)	+	221	256	328	0	Control, not reinoculated			444	0	0
1919	M.	+ (S.T.B.)	+	221	256	335	0	Control, not reinoculated			444	0	0
2312	M.	+ (B.T.)	+	Controls on virus used for reinoculation									
2313	M.	+ (B.S.)	+										

manner. These animals (Nos. 1914 and 1918) both showed numerous generalised lesions following the first intravenous inoculation. Together with 2 controls (Nos. 1911 and 1919) they were treated with arsphenamine, treatment being started on the 221st day and completed on the 256th day. From each of these 4 animals a single popliteal lymph node was removed and transferred to each of 2 normal animals with negative results. The two test animals were then reinoculated intravenously with homologous virus, the other two being reserved as treatment controls. No lesions were observed in the 2 reinoculated rabbits and the Wassermann reaction remained constantly negative. Lymph node transfers were again carried out, 95 days

after the second inoculation. The node of one animal No. 1914, was now found to be infectious. The nodes of the others failed to transmit the infection. It would appear that Rabbit No. 1914 had been infected a second time as a result of the intravenous inoculation, although no lesions had been observed following it. Similar results have been obtained by Uhlenhuth and Grossmann (12).

DISCUSSION

The results of these experiments are sufficiently clear cut to permit of certain generalisations. When rabbits are inoculated with syphilitic virus in such a manner as to produce a primary syphilitic lesion involving the skin of the back, they acquire in time a specific resistance of such a degree that a subsequent inoculation with homologous virus is not followed by the development of a lesion at the site of the second inoculation, whether the latter is subcutaneous, intracutaneous, or intratesticular. The experiments do not disclose if this resistance is of sufficient degree to afford complete protection against the second inoculation when carried out as just described. They do show that, if the second inoculation with homologous virus is made intravenously in rabbits that have had a previous infection with primary lesion involving the skin, an appreciable number, actually 3 in 8, can apparently be successfully reinfected, although they will not exhibit any clinically detectable lesions. The resistance that develops in syphilitic rabbits, therefore, during the course of an infection in which the primary lesion involves the skin, is not absolute, since it is not proof against a second infection introduced directly into the circulation. Nevertheless, it seems permissible to state that this resistant state is imparted to the skin and to the bones, since in the treated animals these tissues do not show lesions on intravenous inoculation, whereas normal animals inoculated in a similar manner exhibit a striking tendency to develop marked lesions of the bones and of the skin.

The experiments reported in detail above demonstrate also that in rabbits a syphilitic infection beginning in the testis is followed in time by the development of a resistant state which attains such a degree that a second intracutaneous inoculation with homologous virus is not followed by any lesions at the site of inoculation.

Again, the experiments demonstrate that in rabbits an intravenous

inoculation of syphilitic virus which is followed by the development of generalised lesions involving skin or bones, is also followed by the development of a resistant state that prevents the occurrence of lesions in the skin or subcutaneous tissue when homologous virus is inoculated in those areas. That the bones share in the resistant state induced in this manner, is indicated by the fact that, after a second intravenous inoculation with homologous virus, no lesions develop in the bones although, as stated above, in normal animals multiple bone lesions are common after intravenous inoculation.

Finally, it would appear that the resistant state developing in the rabbit after intravenous inoculation of syphilitic virus is not always of a degree sufficient to protect the animal against a second infection also introduced by the intravenous route.

In general, the experiments suggest that the state of acquired resistance to syphilis which the infection confers upon rabbits is not a local condition but is widespread, being imparted to skin, bone and testis at least, no matter if the primary lesion involves the skin or testis, or if the inoculation is made directly into the circulating blood. This conception is in complete harmony with the conception of the distribution of the resistant state as developed by Brown and Pearce from a study of the events transpiring during the course of the first infection. Whether the occurrence of clinically detectable lesions during the course of the first infection is essential to the development of this resistant state is not disclosed by the experiments. They do show, however, that the resistance is not always absolute, since intravenous reinoculation may be followed by infection, albeit without lesions. The behavior of a syphilitic rabbit toward reinoculation carried out by intravenous infection is, then, similar to its behaviour if the reinoculation is performed by depositing the virus upon the surface of a granulating wound. As has been shown previously (13), when reinoculations of syphilitic rabbits are carried out in such a manner, there follows a high incidence of second infections, even with homologous virus, although no lesions develop at the site of reinoculation. Both types of inoculation, intravenous injection and deposition of virus on the surface of a granulating wound, can be regarded as favoring the inciting agent rather than the host.

CONCLUSIONS

1. When rabbits are inoculated with syphilitic virus in such a manner as to produce a lesion involving the skin, whether the original inoculation be made subcutaneously, intracutaneously, or by depositing the virus on the surface of a granulating wound, subsequent inoculation with homologous virus after a period of 4 months or more is not followed by the development of lesions, no matter whether the second inoculation be made intratesticularly, subcutaneously, intracutaneously, or intravenously. Intravenous reinoculation under these conditions may be followed by reinfection without lesions.

2. When syphilis develops in rabbits after intratesticular inoculation, a second inoculation with homologous virus by the intracutaneous route after a period of 4 months or more is not followed by the development of any lesions at the site of inoculation.

3. When syphilitic virus is injected intravenously into rabbits, marked and widespread lesions of the skin and bones develop. When these animals are reinoculated with homologous virus after a period of several months, whether by subcutaneous or intracutaneous injection, no lesions develop. However, intravenous injection under these circumstances may be followed by reinfection, but without lesions.

4. The bearing of these facts upon the distribution of acquired resistance in syphilis is discussed.

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A NEW STAIN FOR CELL SURFACES[1]

Preliminary Report

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From the Wilmer Ophthalmological Institute of the Johns Hopkins University and Hospital

The following stain has been found to give a very satisfactory outline of the fibres of the crystalline lens:

(1) Fix in neutral 10 per cent formalin four days.
(2) Wash one day.
(3) Three per cent copper sulphate solution at 37°C., two weeks.
(4) Wash in water five minutes.
(5) Saturated solution of H_2S containing a few drops of 1 per cent HCl, three days.
(6) Cut frozen sections or embed in the usual manner.
(7) Sections may be counterstained with the usual analin dyes if desired.

Tissues other than the lens may be stained in the same way with results resembling those obtained with the familiar silver stains, though much feebler than the silver stains and, therefore, not a useful substitute for them. In bone the calcium is replaced by the copper, and converted into black masses in the sections. For the lens the stain has the advantage over the usual modified Bielschowsky technique, in that there is a complete absence of amorphous precipitate. This does not require any such technical delicacy as is necessary with the silver technique, only moderate care being required to produce uniform results. Counter stains with analin dyes are much more satisfactory after this method than after the silver stains. The method can be used only on blocks of tissue 3 to 4 mm. thick, not on cut sections. Similar results can be obtained by substituting lead acetate for the copper sulphate. The results with this method confirm Kubie's[2] contention that the usual silver stains represent a deposit of precipitate on the surface of cells and fibres rather than a chemical combination of the stain with the tissues.

[1] Read before the Johns Hopkins Research Club, November 21, 1930.
[2] *Arch. Neurol. & Psych.*, 1929, xxii, 135.

BOOK REVIEWS

Modern Methods of Treatment. By LOGAN CLENDENING, M.D. Illustrated. Third Edition. pp. 815. (*St. Louis: The C. V. Mosby Company, 1929.*)

With possibly as much scope as a single individual might reasonably be expected to command, a general survey of the medical treatment of disease is presented. Several minor chapters are written by collaborators, but the great bulk of the work is by the editor. Covering such an extensive field, there are bound to be some inaccuracies and omissions; fortunately, there seem to be surprisingly few of any great consequence. Nearly everyone should find points useful and new in at least some of the chapters on such divergent subjects as physiotherapy, psychotherapy, treatment with biologicals, drugs, diets, etc.

The style is direct, forceful and readable. No doubt is allowed to remain in the reader's mind as to the author's opinion on various topics; it is usually interesting if not always in accord with the generally accepted teachings. Thus, the statement made of polycythemia "It is so rare that reports of results by any method are valueless," seems quite unwarranted.

However, the book contains a fund of information, and should prove invaluable if used with a knowledge of its limitations. There are numerous useful, if homely, diagrams and illustrations, which elucidate the descriptions of various procedures. The diet lists and itemized diets are sensible, and the recipes given seem in line with the present trend toward the culinary education of medical men. In the discussion of drugs as therapeutic agents the names of certain specific preparation found useful in practice should be of value.

The statement that "the best hydrotherapeutic procedures can be carried out in the bath-room of any house in the United States" illustrates the author's praiseworthy desire to simplify methods of treatment which should be available for all, but which perhaps seem unduly complicated to those not familiar with them. The importance of rest, and exercise, as definite and valuable measures in the care of patients is laudably stressed and re-iterated.

At the end of each chapter an introductory bibliography is found, with the titles well-chosen in most instances.

H. R. L.

An Introduction to the Study of the Nervous System. By E. E. HEWER and G. M. SANDES. 103 pages with 55 diagrams. $6.50. (*C. V. Mosby, St. Louis.*)

This book deals with the present day knowledge of neuro-anatomy and neurophysiology in one hundred pages; the information must be, therefore, extremely didactic. The diagrams with which the volume is illustrated are even more diagrammatic than usual, leaving the reader with an idea of multitudinous trolley

lines carrying on the intricate business of reflexes, consciousness and intelligence. The book is well printed.

There would appear to be little value in numerous presentations of neurology in terms of a few disconnected facts; it might be well to consider what worth-while advances could be made in neurological textbooks.

In the study of the highest integrative mechanism of the organism, it is important that more attention should be given to the total individual. The time has passed when it is profitable to examine the motor or sensory systems without keeping the idea of workable reflex arcs constantly in mind. The different portions of the nervous system should be thought of in terms of what they actually can do. In the understanding of the more complex activities of the nervous system this principle is even more useful. It is inadequate to discuss the subject of aphasia in two pages with one diagram.

Perhaps there is no subject which gains more lucidity from the use of really good pictures than neurology. As many structures as possible should be demonstrated in actual dissections of the gross brain, and microscopic material should be used only when absolutely necessary. No books give adequate concepts concerning the third dimension of the brain: this will undoubtedly be achieved in time by the insertion of drawings of models.

An understanding of the phylogeny of the nervous system aids tremendously in clarifying intricate morphological questions. It is time that the rather technical papers of comparative neurologists were made comprehensible for the further elucidation of the subject. Finally, it must be realized that a good textbook should be inspired. None of these aims have been achieved in the present volume.

O. R. L.

The Adrenals, their Physiology, Pathology and Diseases. By MAX A. GOLDZIEHER, Director of Laboratories, United Israel Zion Hospital, Brooklyn, N. Y. 436 pp. $7.50. (*The Macmillan Co., New York, 1929.*)

The author has attempted to give in one volume a complete account of the adrenals, including their morphology, physiology, pathology and their relations to clinical medicine. It is no light task, for the literature is unusually voluminous and full of contradictory statements. This book is not a mere review but gives the author's point of view and his comments on the work of previous writers. His stand is primarily that of the clinician: "the value of observation on man after all should not be minimized even by those who cannot conceive the truth of biological facts if not sanctioned by rabbits and cats."

Perhaps the best idea of the book can be given by summarizing the author's views on a few of the points which still remain controversial. Epinephrin, he believes, either is secreted in an inactive form or becomes (temporarily) inactivated by contact with the blood, and hence the quantity circulating in the blood is sufficient to maintain normal blood pressure, although it cannot be demonstrated by methods at present in use. The effectiveness of epinephrin artificially introduced

differs from that of epinephrin secreted by the gland, as a result of compensatory changes of the antagonists (including the adrenal cortex) and alteration of the electrolytes of the blood. The functions of the cortex are to reduce blood pressure (for this reason and on account of other compensatory processes adrenalectomy does not necessarily alter the pressure) and to reduce the blood cholesterol. It participates in the regulation of heat production and respiration and its rôle in carbohydrate metabolism is probably not negligible, partly because it contains sulphur, an important "radical" in insulin. The nitrogenous waste products accumulating in nephritis stimulate the production of epinephrin which in turn increases their secretion by the kidney.

With regard to diseases, the author finds evidence of acute and chronic hyperfunction and hypofunction. "All the various forms of Bright's disease, with the exception of acute glomerulonephritis, appear to be based on either primary or secondary hyperfunction of the adrenals. The adrenals play a large but not exclusive rôle in the production of essential hypertension."

The book suffers badly from lack of proof-reading and contains many foreign idioms and expressions. In one case a proper name is mispelled so that it is difficult to find the reference in the bibliography. The formula for adrenalin on p. 72 is wrong. The bibliography, which is extensive but not exhaustive, is arranged under various headings, and as some papers might very well appear under several of these, a particular one is sometimes difficult to find; nor is it always easy to be sure that one has the right one, as they are referred to in the text only by the author's name, without a number or year of publication.

G. S. E.

The Great Apes. A study of anthropoid life. By ROBERT A. and ADA W. YERKES. xix + 652 pp. (*Yale University Press, New Haven*, 1929.)

This splendid book deals chiefly, as the sub-title indicates, with what is known of the lives of the anthropoid apes (gibbon, orang-utan, chimpanzee and gorilla) in their natural habitats and in captivity. It is based not only on Professor Yerkes' own extensive investigations of the great anthropoids, but brings together in a most readable and useful manner the researches of naturalists, explorers and other psychologists who have had the opportunity of studying these animals.

The volume is divided into six parts: the first gives an historical survey of our knowledge of the anthropoids; the next four deal with the gibbon, orang-utan, chimpanzee and gorilla, successively, while the final section is devoted mainly to comparisons of the various animals.

Only those anatomical and physiological facts that are necessary for an appreciation of the psychology and natural history of the animals under consideration are given. The morphological data cited are quite adequate for their purpose, and there is, moreover, an excellent bibliography for those with further interests in anthropoid anatomy.

The book represents a piece of most extensive and pains-taking research. In

addition to the text, there are 172 carefully selected and excellently reproduced illustrations. The valuable and well-arranged bibliography contains 674 titles. A detailed index considerably enhances the usefulness of the book.

This timely work should be most welcome to all students of the Primates, whatever their interests.

W. L. S. JR.

Man's Place Among the Mammals. By F. WOOD JONES. 372 + xi pages. 8 vo. (*Longmans, Green & Co., New York and London, 1929.*)

In this interesting and readable volume Professor Wood Jones presents in detail his theory concerning the ancestry of man. The book is divided into three parts, of which the first two, relatively brief, discuss "The evolution of biological thought" and "The evolution of thought concerning man." The remainder of the work deals with "The Primates." It is largely devoted to a more or less systematic treatment of the external and internal characters, habits, paleontology and genetic affinities of the various groups of Primates. The concluding pages deal more specifically with man and with the problem of his ancestry.

The author concludes that the lemurs are not really Primates, but that they are most closely allied to the tree-shrews (Tupaiidae), which are actually primitive lemurs. The true Primates include only the New and Old World monkeys, the gibbons, orang-utan, chimpanzee, gorilla, Tarsius and man. Tarsius is not a lemur, but is the most primitive of the Primates, the sole surviving member of the tarsioid stock which gave rise to the existing groups of Primates. From this early tarsioid stock there arose, on the one hand, the New World or Platyrrhine monkeys, on the other, the Old World or Catarrhine monkeys. The gibbons and the three great apes are rather early offshoots of the Old World stem, and are fundamentally less specialized than the monkeys of the Catarrhine group. Man evolved independently from the early tarsioids, and he is in general more primitive than the Old World monkeys and apes. He therefore does not represent the apex of the Old World stock as is generally assumed. The author, however, finally assumes "the members of the proto-human stock to be forms which had progressed from a primitive tarsioid stage in the general direction of a primitive gibbon" (pp. 356–357).

Professor Wood Jones' conclusions are decidedly unorthodox. Those relating to the relationships of man and the anthropoids are, in particular, inadequately supported. The author attempts to show that man possesses many fundamentally primitive characters which have been lost by monkeys and apes. The structural resemblances between man and anthropoids are regarded as being due to convergence in unrelated forms, yet diametrically opposed reasoning is resorted to in defending the close relationship between anthropoids and Old World monkeys. The author is by no means convincing in his attempt to paint man as an essentially primitive Primate.

A serious defect in the author's presentation and argument lies in his uneven

treatment of the evidence available from studies on various parts of the body. His conclusions appear to be based chiefly upon a consideration of skull characters, an archaic method of treatment. The soft parts receive relatively scant consideration. The references to the musculature, brain and placentation are totally inadequate, and in no sense represent a comprehensive presentation of the existing knowledge on these subjects.

The book, although essentially controversial and a case of special pleading, serves a very definite and stimulating purpose. Professor Wood Jones does not succeed in his presentation of man as a primitive Primate. He does, however, show that the story of man's ancestry has not yet been completely unfolded. Much additional work remains to be done.

W. L. S., Jr.

Hypertension and Nephritis. By A. M. Fishberg. 566 pp. $6.50. (*Lea & Febiger, Philadelphia.*)

In this work, we believe, the author has filled a real need by the coördination of the massive literature on the important and confusing subjects of nephritis and hypertension. The table of contents, which indicates how thoroughly the hypertensive and renal diseases are covered, is a promise that the rest of the book fulfils. The basis of the work is the pathological anatomy as begun by Volhard and Fahr rather than the functional aberrations, although the latter are well discussed. The complete collection of references on every matter treated is impressive and to those interested in the subject the book would be worth while for its large and well selected bibliography. Great care is manifested in the arrangement and presentation of the material and this results in a degree of cosmos that is most welcome in this confusing field. To do this there has been an orderly selection of facts and ideas, and many dissonant theories have been put aside. While the author's choice has been sound and his discussion of differing ideas is fair, one hopes that fact and theory will not be confused or the subjects be made to seem more simple than they are. Since this book is primarily a review of literature and just because it is so up to date, it contains much that is not final. It is probable that the author's classification of arterial lesions, which are rarely as distinct as his written description, could not be generally employed by pathologists.

In contrast to those parts of the book which are of necessity "labile," the clinical descriptions are accurately drawn and the important criteria in history, physical diagnosis and laboratory studies are emphasized. This presentation appears eminently serviceable and the book as a whole is well written and very readable. In brief, the full and up to date review of literature, the clear clinical pictures, the emphasis on selection rather than quantity of laboratory work, conservatism and good judgment in the sections on treatment should all happily fulfil the author's purpose "to summarize for the practitioner the information that practice, clinic and laboratory have yielded regarding the hypertensive and renal diseases."

F. D. W. L.

Principles of Surgery for Nurses. By M. S. WOOLF, M.A., B.Sc., M.R.C.S. (Eng.), L.R.C.P. (Lond.). Second Edition, Revised. 355 pp. (*W. G. Saunders Co., Philadelphia, 1930.*)

'This [relatively] small volume,' as the author tells us in the preface, 'has arisen through the endeavor to meet the demands of the nurse for a simple statement and explanation of surgical affections.' The book is written in a readable style, the illustrations are good and there is an excellent index. It should be very helpful for students in surgical nursing.

D. B.

The Determination of Hydrogen Ions. By W. M. CLARK. Third Edition, Pp. XVI and 717. Price $6.50. (*The Williams & Wilkins Co., Baltimore, Md., 1928.*)

It is a remarkable phenomenon in the madly prolific world of textbooks that in the decade since the appearance of Professor Clark's epoch-making *Hydrogen Ions* it has retained its peak position as the one authoritative work in its field. This is all the more remarkable if one bears in mind the fact that the great popularity of this book rests not on any deliberate attempt at popularization of intrinsically complicated thermodynamic concepts, but distinctly upon brilliantly lucid style and honest exposition of the theory, and above everything else, perhaps, upon the happy faculty of the author to cull from the maze and welter of the enormous literature the significant and well established facts. A glance at the steeply mounting curve, in the preface, of the annual number of published papers having direct bearing upon the theory and practice of hydrogen-ion determination brings forcefully to the attention the herculean task which confronted the author in the preparation of the last edition. That this task was successfully surmounted without a great increase in the size of the book and yet ample space devoted to a beautifully adequate treatment of the latest developments is a matter of great amazement to the reviewer. The reader familiar with the previous editions will find in this new edition accurate statement and comprehensive discussion of such newly important topics as (new chapters): Application of Spectrophotometry to Indicators, Changes of Free Energy, Oxidation—Reduction Potentials, Quinhydrone and Similar Half-Cells, The Glass Electrode, The Theory of Debye-Hückel. The appendices contain much added useful data, including an extensive list of carefully stated definitions of terms and units. The chapter on applications and the bibliography are necessarily but critically enlarged to encompass the more important references from 1922 to 1928. The appearance and typography of the book remain on the high level established for the preceding editions.

The "Fellow Workers in the Biological Sciences" to whom this treatise is so truly and generously dedicated owe an inestimable debt of gratitude to the author which may only be repaid in increasingly better "Hewn Stone" and in statelier "Unseen Spires" of the future.

W. A. P.

BOOKS RECEIVED

Peptic Ulcer. [Clinical Roentgenology, With Case Histories]. By JACOB BUCKSTEIN M.D. Introduction by Harold E. Santee, M.D. Two hundred and forty roentgen-ray studies and forty-seven clinical illustrations. 1930. 4°. 388 pages. Annals of Roentgenology, Vol. X. Paul B. Hoeber, Inc., New York. $12.00.

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The Psychoanalysis of the Total Personality. The Application of Freud's Theory of the Ego to the Neuroses. By FRANZ ALEXANDER, M.D. Authorized English Translation by Bernard Glueck, M.D. and Bertram D. Lewin, M.D. With a Prefatory Note by A. A. Brill, M.D. 1930. 8°. 176 pages. Nervous and Mental Disease Publishing Company, Washington, D. C.

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